Neurosteroid migration to intracellular compartments reduces steroid concentration in the membrane and diminishes GABA-A receptor potentiation

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Neurosteroids are potent modulators of GABA-A receptors. We have examined the time course of development of potentiation of $\alpha1\beta2\gamma2$L GABA-A receptors during coapplication of GABA and an endogenous neurosteroid (3α,5α)-3-hydroxypregnan-20-one (3α5αP). The simultaneous application of 3α5αP with 5 μM GABA resulted in a biphasic rising phase of current with time constants of 50–60 ms for the rapid phase and 0.3–3 s for the slow phase. The properties of the rapid phase were similar at all steroid concentrations but the time constant of the slower phase became successively shorter as the steroid concentration was increased. Potentiation developed very rapidly (τ = 130 ms) when cells were preincubated with 300 nM 3α5αP before application of GABA + 3α5αP, and in outside-out patch recordings, suggesting that steroid diffusion to intracellular compartments competes with receptor potentiation by depleting the cell membrane of steroid. Very low steroid concentrations (3–5 nM) potentiated GABA responses but the effects took minutes to develop. Intracellular accumulation of a fluorescent steroid analogue followed a similar time course, suggesting that slow potentiation results from slow accumulation within plasma membrane rather than indirect effects, such as activation of second messenger systems. In cell-attached single-channel recordings, where 3α5αP is normally applied through the pipette solution, addition of steroid to the bath solution dramatically shifted the steroid potentiation concentration–effect curve to lower steroid concentrations. We propose that bath-supplied steroid compensates for the diffusion of pipette-supplied steroid out of the patch to the rest of the cell membrane and/or intracellular compartments. The findings suggest that previous studies overestimate the minimum concentration of steroid capable of potentiating GABA actions at GABA-A receptors. The results have implications for the physiological role of endogenous neurosteroids.

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Neurosteroids, defined as steroids synthesized by neurons and glia in the central nervous system, are among the most potent GABA-A receptor modulators. Depending on the receptor subunit combination or the type of neurons studied, channel potentiation can be observed at steroid concentrations corresponding to tens or hundreds of nanomolar. Despite their potency, modulation by endogenous steroids is often disregarded because the physiological levels of most neurosteroids are considered to be lower than those capable of producing GABA-A receptor modulation. For example, the EC_{50} for 3α5αP actions on the most prevalent mammalian GABA-A receptor subtype ($\alpha1\beta2\gamma2$) is 100 nM or more (Maitra & Reynolds, 1998; Shu *et al.* 2004; Weir *et al.* 2004; Akk *et al.* 2005). In contrast, the estimates for total brain concentration of 3α5αP are generally at or below tens of nanomolar (Cheney *et al.* 1995; Weill-Engerer *et al.* 2002), making it unlikely that the $\alpha1\beta2\gamma2$ GABA-A receptor is under persistent steroid tone under physiological conditions.

Previous studies have shown that steroid actions are slower to develop than typical ligand–receptor interactions (e.g. Liu *et al.* 2002; Wohlfarth *et al.* 2002; Shu *et al.* 2004) although the underlying reasons for this are unclear. Two recent studies proposed that slow accumulation of steroids in a non-aqueous reservoir(s) underlies the slow kinetics of direct activation and receptor potentiation (Shu *et al.* 2004; Akk *et al.* 2005) but the role of the plasma

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membrane *versus* intracellular hydrophobic reservoirs in slowing steroid actions remained unclear. An earlier study (Liu *et al.* 2002) described multiphasic responses to $3\alpha5\alpha$P in embryonic rat hippocampal neurons, and found that pertussis toxin treatment affected the responses suggesting that a pathway involving second messengers is involved in GABA-A receptor response to steroid application.

In this work, we examined the onset kinetics of steroid potentiation, and the role of intracellular compartments in limiting the electrophysiological response. Our data indicate that intracellular compartments drain steroid from the plasma membrane during drug application, slowing the onset kinetics in macroscopic experiments. In cell-attached single-channel recordings, the outflow of steroid from the cell membrane reduces the steroid concentration in the patch. As a result, steroid-mediated potentiation is underestimated in both single-channel and macroscopic recordings in many experimental protocols. The data support the physiological role of endogenous neurosteroids, and suggest that significant GABA-A receptor potentiation may occur under normal physiological conditions *in vivo*.

Methods

The electrophysiological experiments were conducted on HEK293 cells transiently expressing rat $\alpha1\beta2\gamma2$L GABA-A receptors. The subunit cDNAs were subcloned into the pcDNA3 expression vector (Invitrogen, Carlsbad, CA, USA) and expressed in HEK cells using a calcium phosphate precipitation-based transient transfection technique (Akk, 2002). The amino-terminal end of the $\alpha1$ subunit contains the FLAG epitope tag, whose presence is without effect on channel kinetics (Ueno *et al.* 1996).

The electrophysiological experiments were carried out using cell-attached single-channel patch clamp, whole-cell voltage clamp, and excised outside-out recordings. The bath solution contained (mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose and 10 Hepes, pH 7.4. In single-channel recordings, the pipette solution contained (mM): 120 NaCl, 5 KCl, 10 $MgCl_2$, 0.1 $CaCl_2$, 20 tetraethylammonium, 5 4-aminopyridine, 10 glucose, 10 Hepes; pH 7.4. In macroscopic recordings, the pipette solution contained (mM): 140 CsCl, 4 NaCl, 4 $MgCl_2$, 0.5 $CaCl_2$, 5 EGTA and 10 Hepes, pH 7.4.

The agonist (GABA) was added to the pipette solution in single-channel recordings, or applied through the bath using an SF-77B fast perfusion stepper system (Warner Instruments, Hamden, CT, USA) in macroscopic experiments. The steroid was applied through the bath in macroscopic experiments, and through the pipette solution or simultaneously through the pipette and bath solutions in single-channel experiments. The solution exchange time for macroscopic experiments, estimated from switching the bath solution from 100% to 75% Ringer solution, was 2–3 ms when measured with open tip, and 10–20 ms when measured with a typical HEK cell. In single-channel experiments with bath-applied steroid, a dish with cells was first incubated with a given concentration of $3\alpha5\alpha$P for approximately 15 min. The recordings were then carried out during the next 2–3 h while the steroid remained in the bath. The steroids were initially dissolved in DMSO at 10 mM concentration, and diluted immediately before the experiment. Previous whole-cell recordings have shown that the presence of DMSO at concentrations up to 0.3% is without effect on channel activation by GABA (Li *et al.* 2007).

The GABA concentration in single-channel experiments was 50 μM, a concentration which corresponds to EC_{40} in the open probability concentration–effect curve (Steinbach & Akk, 2001). The pipette potential was held at +60 to +80 mV, which translates to a −120 to −100 mV potential difference across the patch membrane. The channel activity was recorded using an Axopatch 200B amplifier, low-pass filtered at 10 kHz, and acquired with a Digidata 1320 series interface at 50 kHz using pCLAMP software (Molecular Devices, Union City, CA, USA). The key features of the analysis of single-channel currents have been described in detail previously (Steinbach & Akk, 2001; Akk *et al.* 2001, 2004). In brief, the analysis was carried out on clusters, i.e. episodes of intense activity originating from the activation of a single ion channel, or portions of clusters containing no overlapping currents. The currents were low-pass filtered at 2–3 kHz, and idealized using the segmented-k-means algorithm (Qin *et al.* 1996). The open and closed times were estimated from the idealized currents using a maximum likelihood method which incorporates a correction for missed events (QuB Suite; www.qub.buffalo.edu).

Most macroscopic recordings were carried out with 5 μM GABA, a concentration which produces approximately 25% of the maximal peak current (Li *et al.* 2006). The cells were clamped at −60 mV. The membrane currents were recorded using an Axopatch 200B amplifier, low-pass filtered at 2 kHz and acquired at 10 kHz. The data were stored on a PC hard drive for further analysis. The analysis of whole-cell currents was carried out as previously described (Li *et al.* 2006), and was aimed at determining the peak amplitude and/or rising phase kinetics.

For fluorescence studies, untransfected HEK cells were imaged by conventional epi-fluorescence during application of a neuroactive steroid analogue tagged with a (7-nitro-2,1,3-benzoxadiazol-4-yl)amino (NBD) group at carbon 11 (C11-NBD $3\alpha5\alpha$P; Akk *et al.* 2005). C11-NBD $3\alpha5\alpha$P at 5 nM was applied by bath exchange (Fig. 4); 500 nM was applied by rapid perfusion (Fig. 5). Emission at

535 nm was collected and analysed using a 60×, 1.45 NA. objective (Fig. 4) or 40×, 0.8 NA. objective (Fig. 5) and CoolSnap ES2 camera (Photometrics, Tucson, AZ, USA). Extreme care was taken at the low drug concentrations (Fig. 4) to prevent photobleaching. Heavy neutral density filtering was employed, and light exposure was limited to 100 ms per image.

Results

Onset kinetics of steroid potentiation of whole-cell responses exhibits two phases

Visual examination of macroscopic currents recorded in the presence of GABA and varying concentrations of 3α5αP shows that the current rising phase contained two components. The initial phase was relatively fast, with a time constant and amplitude in most cases similar to those observed in the presence of GABA alone. The second phase developed more slowly and had a time constant that inversely scaled with steroid concentration.

We show in Fig. 1*A* a sample recording from a cell exposed to 5 μM GABA alone, or along with 50, 100, 300 or 1000 nM 3α5αP. It is evident from the illustration that the presence of the steroid resulted in an additional component in the rising phase of the current. The time constant of the fast component was largely unaffected by the presence of steroid. In contrast, the slow component became more rapid as the steroid concentration was increased. Averaged from four cells, the fast component had a time constant of 55 ± 9 ms (mean $\pm$ S.D.), 54 ± 11 ms, 61 ± 29 ms, 63 ± 19 ms and 66 ± 18 ms with GABA alone, and in the presence of 20, 50, 100 or 300 nM 3α5αP, respectively. The slow component had a time constant of 2.5 ± 0.5 s, 2.8 ± 1.3 s, 1.1 ± 0.4 s, 0.71 ± 0.31 s and 0.25 ± 0.07 s with 5 μM GABA + 20, 50, 100, 300 or 1000 nM 3α5αP, respectively. The slow component was not present in the absence of steroid, and the fast component could not be resolved when GABA and 1000 nM 3α5αP were coapplied.

The steroid concentration dependence of the second, slower phase suggests several potential underlying

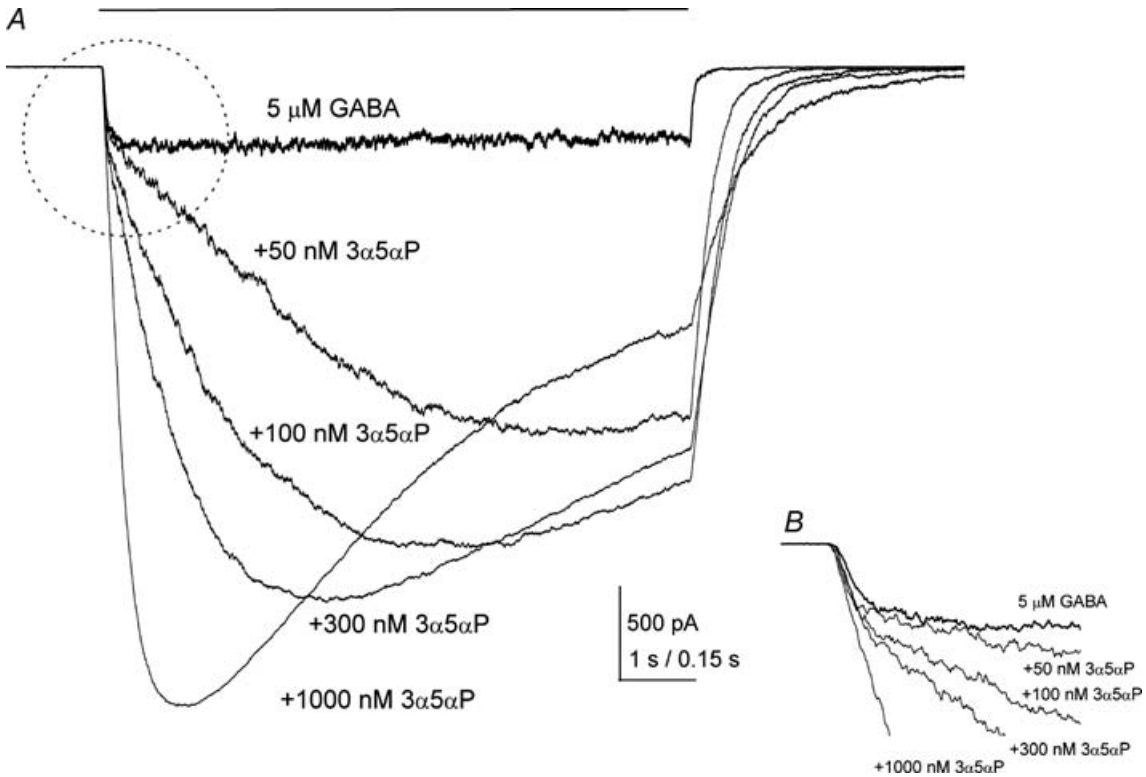


Figure 1. The kinetics of current development during channel potentiation by 3α5αP
A, the rate of current development depends on the concentration of 3α5αP. Representative macroscopic current traces from a HEK cell expressing α1β2γ2L GABA-A receptors. The receptors were activated by 8 s applications of 5 μM GABA or GABA plus 50–1000 nM 3α5αP. Successive applications were separated by 60 s washout periods. The increase in the steroid concentration led to an increase in the peak response and a faster rise time. *B*, the area shown by a dotted circle in *A* is shown at a higher time resolution. In most cells, the increase in the concentration of steroid did not affect the time course or amplitude of the first, faster component of current development.

mechanisms. First, the slow phase may result from slow steroid binding to the receptor. At low steroid concentrations, the binding rate (k_{on}[steroid]) is low and potentiation develops slowly. As the steroid concentration is increased the rate with which the steroid binds to the receptor increases and potentiation develops more quickly. Finally, when a saturating concentration of 3α5αP is coapplied with GABA, the rate of current development becomes limited by the system's solution exchange time or receptor activation kinetics. Alternatively, the data can be accounted for by the presence of a slowly equilibrating compartment, e.g. intracellular membrane fractions, which initially act by depleting the cell membrane of the steroid. The diffusion of steroid out of the cell membrane into such compartments reduces the effective steroid concentration available to the membrane-localized receptor, and, as a result, thwarts receptor potentiation until equilibrium is reached. Finally, the slow phase may be linked to activation of second messenger systems.

Preincubation with steroid removes the slow phase of steroid potentiation

Because GABA and steroid were coapplied in preceding experiments, we first addressed whether receptor activation influences the slow onset of steroid potentiation. We tested whether preexposure to steroid, in the absence of GABA, can be used to equilibrate the binding sites or the compartments where the steroid accumulates, and whether this modifies the time course of the following jump to GABA + steroid. In this experiment, we preexposed the cell to a relatively low concentration (300 nM) of 3α5αP for 60 s (Fig. 2*A*). At this steroid concentration, essentially no direct activation could be observed, although at the end of the 60 s steroid application some increased baseline noise, suggestive of channel activity, was usually evident. At the end of the steroid incubation, the solution washing the cell was switched to 5 μM GABA + 300 nM 3α5αP. In this experimental protocol, the slow phase of steroid potentiation was not present.

Although the results of this experiment cannot be used to distinguish between the alternative possibilities of slow binding of the steroid to the receptor and slow equilibration of the cell with steroid, the results demonstrate that equilibration of steroid sites (on the receptor or in the intracellular compartments), or the activation of the second messenger system can occur in the absence of receptor activation.

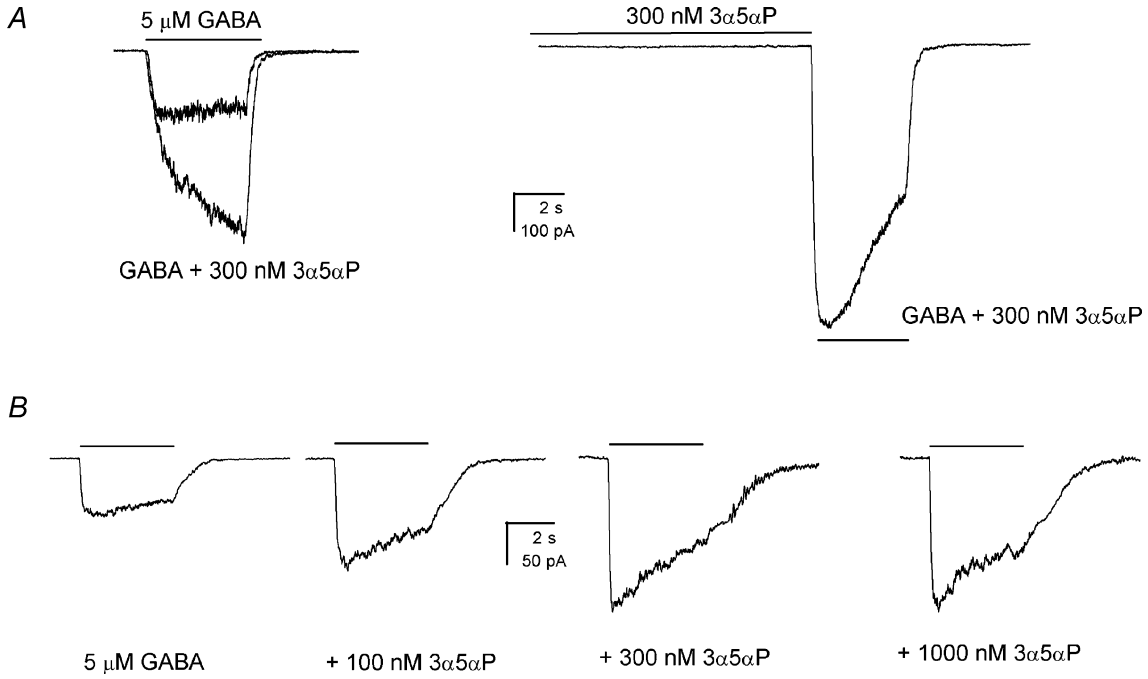


Figure 2. The slow phase is removed by preincubation with steroid and patch excision
A, preincubation with 3α5αP modifies the shape of current development for subsequent application of GABA + steroid. A cell exposed to 5 μM GABA and 300 nM 3α5αP shows the characteristic slow rise phase (left panel). In the same cell, preincubation with 300 nM 3α5αP for 60 s resulted in the loss of the slow rising phase (right panel), presumably because of filling of compartments and/or sites to which the steroid may bind. *B*, an outside-out patch was exposed to 5 μM GABA alone or GABA in the presence of 100–1000 nM 3α5αP. The applications lasted for 4 s, and were separated by 30 s washout periods. The presence of steroid led to an increase in peak current without introducing the second, slow rising phase. The time constants for the rising phase are as follows: 5 μM GABA, 73 ms; GABA + 100 nM 3α5αP, 88 ms; GABA + 300 nM 3α5αP, 77 ms; GABA + 1000 nM 3α5αP, 74 ms.

Patch excision removes the slow phase of steroid potentiation

If intracellular compartments act by initially depleting the cell membrane of steroid, and are responsible for the slow current development during exposure to GABA + steroid, then removal of such compartments should abolish the second, slow phase. We tested this hypothesis by examining channel potentiation in excised outside-out patches. We reasoned that the lack of intracellular membranous components would remove the drainage of steroid into the cell, and thus potentiated currents should have a simple, monophasic time course, and conversely, if slow steroid binding to the receptor is responsible for the slow phase of potentiation, then patch excision should have minimal effect on the kinetics of current development.

The receptors were activated by 5 μM GABA, and potentiation was examined at 100–1000 nM 3α5αP. A sample recording from an outside-out patch is shown in Fig. 2*B*. In this example, and in patches from five other cells studied, the current rise times were generally fast and lacked the slower phase that is prominent in whole-cell recordings. The remaining time constant was similar in its duration (see Fig. 2*B*) to the faster component in whole-cell recordings. This finding strongly suggests that steroid movement into intracellular compartments, not slow binding to the receptor, underlies the second, slow phase in potentiation time courses.

The relative amount of potentiation was similar in outside-out patches and whole-cell recordings. In excised patches ($n = 6$ patches), exposure to 1000 nM 3α5αP resulted in 412 $\pm$ 148% potentiation (response to 5 μM GABA alone was considered 100%). In whole-cell recordings, 2 and 20 s drug applications led to a maximal potentiation of 410 $\pm$ 48% and 472 $\pm$ 10%, respectively (Fig. 3*C*).

Macroscopic concentration–effect properties depend on application duration

The data presented in Fig. 1 show that potentiation develops slowly upon the application of steroid. This suggests that during brief drug applications the degree of potentiation may be underestimated because the current does not reach its peak. Thus, the steroid potentiation concentration–effect relationship could be dependent on the application duration, with a higher steroid potency observed when drug applications are prolonged.

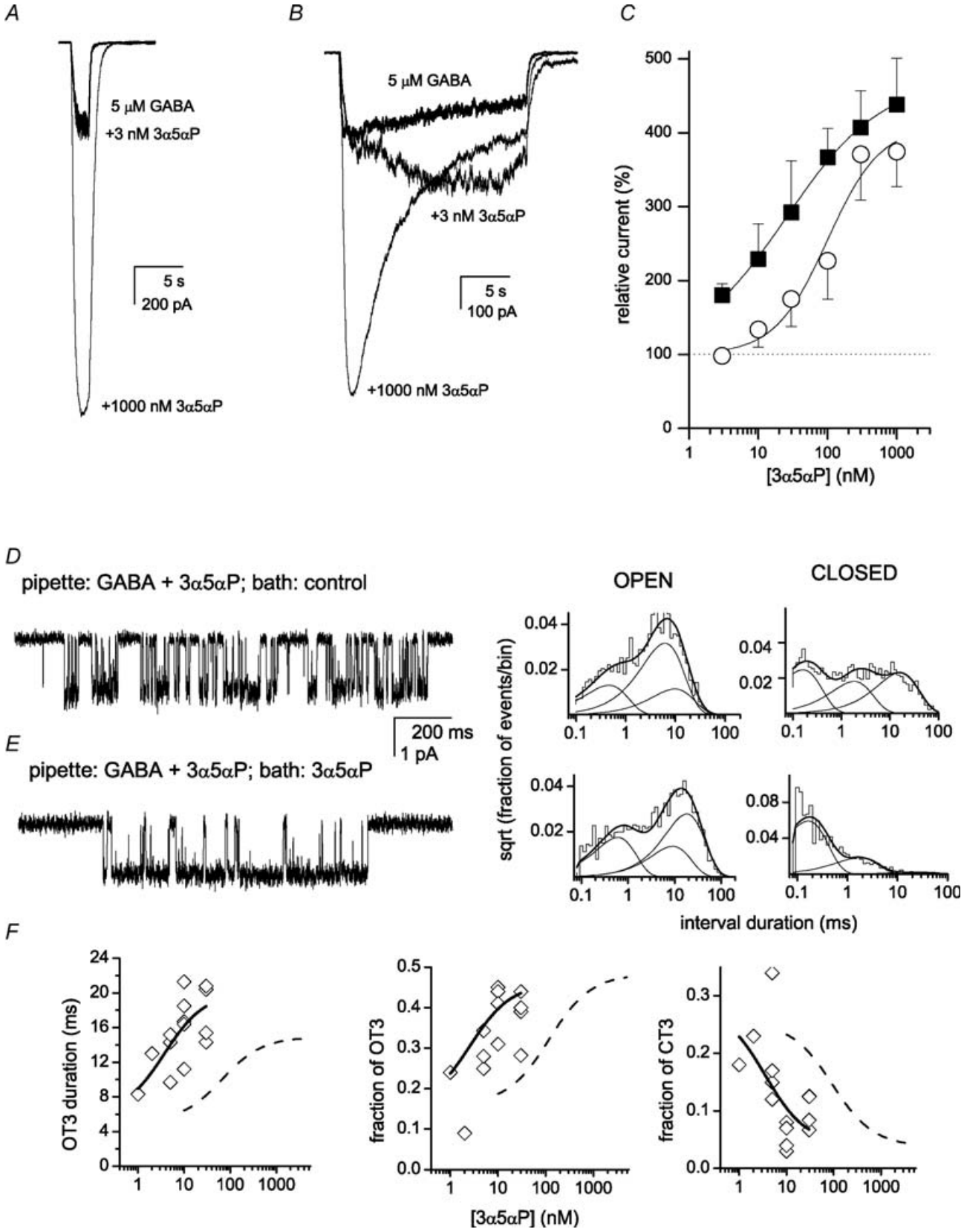
We tested this hypothesis by examining receptor potentiation by 3α5αP when 20 s drug applications were used *versus* 2 s applications. Sample current traces and the corresponding concentration–effect relationships are shown in Fig. 3*A*–*C*. The results demonstrate that the application duration has a striking effect on the current response. Potentiation was much more robust when prolonged drug applications were employed (Fig. 3*A* *versus* Fig. 3*B*). The duration of drug application also affected the potentiation concentration–effect relationship (Fig. 3*C*). The midpoint of the potentiation curve for 3α5αP was 100 nM when 2 s drug applications were employed, and 26 nM with 20 s applications. The shape of the concentration–effect curve at low steroid concentrations would likely be affected even further with applications lasting several minutes.

Single-channel potentiation is affected by steroid diffusion out of the patch membrane

The results from macroscopic recordings suggest that intracellular compartments efficiently drain the steroid from the cell membrane, and by doing so reduce the concentration of steroid available to bind to the cell surface-located receptors affecting macroscopic potentiation. This raises a possibility that steroid diffusion may similarly influence channel potentiation in single-channel recordings. When studying single-channel currents in the cell-attached configuration, the agonist and modulators are typically applied through the patch pipette so that only the extracellular surface of the patch membrane is exposed to the drugs. With efficient drainage of steroid out of the patch to the surrounding cell membrane and/or into the intracellular compartments, the steroid concentration in the patch membrane may be much lower than what is dictated by the steroid dose in the pipette solution. This could lead to a rightward shift in the steroid concentration–effect properties because extra steroid would have to be supplied to the patch membrane to compensate for the loss due to steroid diffusion out of the patch membrane.

To test this hypothesis, we compared 3α5αP-mediated potentiation under two conditions. In the first case, the steroid was applied solely through the pipette solution. This is a typical experimental setting for cell-attached recordings. In the second setting, the steroid was present both in the pipette solution and in the bath. This experiment is based on the idea that if the same concentration of steroid is present in the bath and in the pipette solution, then, following some equilibration period, the steroid concentration in the intracellular compartments would be the same as in the patch membrane. Consequently, there would be no net movement of steroid out of the patch membrane.

In Fig. 3*D* and *E*, we show sample single-channel recordings obtained in the presence of 50 μM GABA and 10 nM 3α5αP. The steroid was applied solely through the patch pipette (Fig. 3*D*) or through the pipette and bath solutions (Fig. 3*E*). In agreement with our previous findings (the dashed lines in Fig. 3*F*; Akk *et al.* 2005), the application of 10 nM 3α5αP solely through the pipette



solution was ineffective at modulating receptor activity. In contrast, when the steroid was present in the bath solution, in addition to the pipette solution, 10 nM 3α5αP caused significant potentiation in channel open probability. A summary of these data is presented in Table 1.

Exposure of the GABA-A receptor to many potentiating steroids affects three kinetic parameters of single-channel currents: the duration and prevalence of the longest open time component are increased, and the prevalence of the longest-lived intracluster closed time component is decreased (Akk *et al.* 2004, 2005). Full concentration–effect relationships for these kinetic parameters are shown in Fig. 3*F*. The presence of steroid in the bath led to a robust leftward shift in the concentration–effect curves. The concentration of steroid producing half-maximal effect was ~100 nM when steroid was present only in the pipette solution, and ~3 nM when steroid was in the pipette and bath solutions.

Activity of steroids at very low concentrations correlates with intracellular accumulation

An implication of slow onset is that previous studies may have overestimated steroid concentrations necessary to potentiate GABA-A receptor function. Previous studies have suggested that concentrations below 10 nM 3α5αP are ineffective at modulating $\alpha1\beta2\gamma2$L GABA-A receptor function (e.g. Shu *et al.* 2004; Akk *et al.* 2007). By contrast, we found that concentrations as low as 3 nM 3α5αP effectively potentiated GABA responses in transfected HEK cells, but the effects were slow to develop (Fig. 3*B*).

Slowly developing potentiation may be a function of steroid-activated second messenger systems that interact with GABA-A receptor function (Liu *et al.* 2002; Wegner *et al.* 2007) rather than a direct effect of steroid that accumulates slowly in the cell. Although steroid potentiation cannot rise exclusively from second messengers because potentiation occurs in excised patches (Fig. 2*B*), second messengers could conceivably contribute specifically to the slow component of whole-cell potentiation. To test whether intracellular steroid accumulation is likely to account for slow potentiation of GABA-gated currents by low concentrations of steroids, we compared the time course for potentiation with the time course for imaged accumulation of a fluorescent neuroactive steroid analogue C11-NBD 3α5αP. This fluorescent steroid acts as a potentiator of the $\alpha1\beta2\gamma2$L GABA-A receptor, although it has a lower potency than its parental compound, 3α5αP (Akk *et al.* 2005). Fluorescence measurements of accumulation of 5 nM C11-NBD 3α5αP in a HEK cell are shown in Fig. 4*A*. An exponential fit to averaged signals from three cells gave a time constant of accumulation of 1.9 min. Concentrations of C11-NBD 3α5αP up to 300 nM yielded extremely slow fluorescence accumulation, with time constants > 30 s when steroid was continuously applied. When we examined potentiation by the fluorescent steroid, we found that C11-NBD 3α5αP exhibits slow potentiation. Potentiation in the presence of 10 nM C11-NBD 3α5αP developed with a time constant of

Figure 3. Steroid concentration–effect properties depend on equilibration of the cell with steroid
A, whole-cell currents were elicited by 5 μM GABA alone or GABA in the presence of 3 nM or 1000 nM 3α5αP. The drug applications were 2 s in duration, and separated from successive recordings with 30 s washout periods. Exposure to 3 nM 3α5αP had no discernible effect on current response. *B*, whole-cell currents were elicited by 5 μM GABA alone or GABA in the presence of 3 nM or 1000 nM 3α5αP. The drug applications were 20 s in duration, and separated from successive recordings with 90 s washout periods. Exposure to 3 nM 3α5αP roughly doubled the peak current. *C*, steroid potentiation concentration–effect curves at 5 μM GABA were measured using 2 s (○) or 20 s (■) drug applications. Each symbol represents the mean value from data from 3 to 5 cells. The curves were fitted to: relative current = maximal potentiation/$(1 + (EC_{50}/[GABA])^{n_H})$. Offset was fixed at 100%. The best-fit parameters for 2 s applications are: maximal potentiation = 410 ± 48%, EC_{50} = 100 ± 44 nM, n_H = 1.14 ± 0.39. The best-fit parameters for 20 s applications are: maximal potentiation = 472 ± 10%, EC_{50} = 26 ± 3 nM, n_H = 0.63 ± 0.04. The difference in potentiation by 1000 nM 3α5αP is not significantly different for 2 s *versus* 20 s applications. *D*, a sample single-channel cluster obtained with 50 μM GABA and 10 nM 3α5αP in the pipette. The open and closed time histograms were fitted to three exponentials. The open times were: 0.42 ms (24%), 5.7 ms (57%) and 9.0 ms (20%). The closed times were: 0.15 ms (38%), 1.7 ms (28%) and 14.7 ms (34%). *E*, a sample single-channel cluster obtained with 50 μM GABA and 10 nM 3α5αP in the pipette, and 10 nM 3α5αP in the bath solution. The open times were: 0.55 ms (31%), 8.3 ms (24%) and 16.4 ms (45%). The closed times were: 0.15 ms (74%), 1.5 ms (23%) and 20.0 ms (3%). *F*, steroid concentration–effect curves for the duration of OT3, fraction of OT3 and fraction of CT3. The receptors were activated by 50 μM GABA. The control data (shown with the dashed line) are from Akk *et al.* (2005), and apply to data obtained with steroid in the pipette but not in the bath. Each symbol stands for data from one patch obtained under the condition where steroid, at the same concentration as in the pipette solution, was also present in the bath solution. The curves were fitted using the equation $Y([steroid]) = Y_0 + (Y_{max} - Y_0)[steroid]/([steroid] + EC_{50})$. The EC_{50} values for the control data (steroid only in the pipette) are: 66 nM (OT3 duration), 119 nM (fraction of OT3) and 94 nM (fraction of CT3). The EC_{50} values for the data obtained with steroid in the pipettete and the bath solutions are: 3.8 ± 2.5 nM (OT3 duration), 2.6 ± 2.8 nM (fraction of OT3) and 3.5 ± 6.5 nM (fraction of CT3).

Table 1. The summary of single-channel kinetic analysis of currents from the $\alpha1\beta2\gamma2L$ GABA-A receptor exposed to GABA and 3α5αP

Pipette	Bath	OT1 (ms)	Fraction of OT1	OT2 (ms)	Fraction of OT2	OT3 (ms)	Fraction of OT3	*n*
50 μM GABA	—	0.28 ± 0.05	0.22 ± 0.02	3.0 ± 0.7	0.65 ± 0.06	7.3 ± 3.2	0.13 ± 0.07	4
50 μM GABA + 10 nM 3α5αP	—	0.30 ± 0.09	0.25 ± 0.08	3.6 ± 1.3	0.49 ± 0.13	8.8 ± 1.0†	0.26 ± 0.15†	5
50 μM GABA + 1000 nM 3α5αP	—	0.41 ± 0.04	0.39 ± 0.07	2.4 ± 0.9	0.23 ± 0.03	14.1 ± 2.1*	0.38 ± 0.04*	3
50 μM GABA + 10 nM 3α5αP	10 nM 3α5αP	0.41 ± 0.11	0.27 ± 0.04	5.4 ± 3.1	0.28 ± 0.08	17.6 ± 3.8***,***,†	0.45 ± 0.09**,†,†	6
Pipette	**Bath**	**CT1 (ms)**	**Fraction of CT1**	**CT2 (ms)**	**Fraction of CT2**	**CT3 (ms)**	**Fraction of CT3**	***n***
50 μM GABA	—	0.15 ± 0.01	0.60 ± 0.10	1.5 ± 0.2	0.13 ± 0.05	14.4 ± 4.2	0.27 ± 0.06	4
50 μM GABA + 10 nM 3α5αP	—	0.16 ± 0.03	0.50 ± 0.11	1.5 ± 0.2	0.26 ± 0.06	11.1 ± 2.3	0.23 ± 0.07†	5
50 μM GABA + 1000 nM 3α5αP	—	0.22 ± 0.04	0.64 ± 0.12	1.4 ± 0.2	0.30 ± 0.10	14.3 ± 1.2	0.05 ± 0.01***	3
50 μM GABA + 10 nM 3α5αP	10 nM 3α5αP	0.15 ± 0.02	0.70 ± 0.06	1.5 ± 0.2	0.25 ± 0.07	13.4 ± 3.8	0.05 ± 0.02***,***,†	6

The mean durations (OT1-3, CT1-3) and relative contributions (Fractions of OT1-3, Fractions of CT1-3) for the three open and closed time components are shown. The presence of high concentrations of 3α5αP affects the duration of OT3, the fraction of OT3, and the fraction of CT3 (Akk *et al.* 2005). Potentiation is essentially saturated in the presence of 1000 nM 3α5αP demonstrating the extent of possible increases in the duration and fraction of OT3, and the decrease in the fraction of CT3. At 10 nM, 3α5αP, when applied solely through the pipette solution, has a weak, non-significant effect on these parameters. When applied simultaneously through the pipette and bath solutions, exposure to 10 nM 3α5αP results in strong potentiation of single-channel currents. The kinetic effects for pipette- and bath-applied 10 nM 3α5αP are not different from the effect of pipette-applied 1000 nM 3α5αP. Statistical analysis was carried out using ANOVA with Bonferroni correction (Systat 7.0; Systat Software, Inc., Point Richmond, CA, USA). For pipette-applied 3α5αP (10 nM or 1000 nM), the significance level applies to comparison to no steroid (control) condition. For pipette- and bath-applied 10 nM 3α5αP, the significance levels apply to comparison to control condition, to pipette-applied 10 nM 3α5αP, and to pipette-applied 1000 nM 3α5αP, respectively. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, †not significant.

1.6 min (Fig. 4*B*), similar to the accumulation of cellular fluorescence in Fig. 4*A*.

In cells exposed to C11-NBD 3α5αP, the time course for the development of fluorescence was generally slower than the time course for the development of potentiation in cells exposed to the parent compound 3α5αP (for example, compare Fig. 3*B* to Fig. 4*A*), and even though the time courses for potentiation and fluorescence by C11-NBD 3α5αP were highly consistent with each other, the lack of consistency between labelled and unlabelled steroids raises the possibility that labelling itself may have affected the distribution of steroid in the membrane and that the much slower time course seen with the labelled steroid is due to additional mechanisms. To address this issue, we examined the development of potentiation by 3α5αP under conditions similar to those used in imaging experiments (> 5 min applications). In order to avoid additional slow changes in response due to channel desensitization we used 0.5 μM GABA (EC_5) to activate the receptors. As expected, exposure to 3 nM 3α5αP resulted in slowly developing potentiation with a time constant of 2.5 min (Fig. 4*C*). This is gratifyingly similar to the potentiation and fluorescence development time constants for C11-NBD 3α5αP, and indicates that both steroids act principally similarly.

Our hypothesis predicts that steroid should accumulate in the plasma membrane first, with subsequent redistribution toward intracellular pools. Therefore, we also attempted to image steroid accumulation in the cell membrane and cell interior separately, to assay for compartmentalization of the steroid. To do that, we conducted fluorescence measurements at 200 ms intervals during a brief 2 s perfusion of a higher concentration (0.5 μM) of C11-NBD 3α5αP. We analysed the change in fluorescence in a peri-membrane region and an intracellular region in 11 cells during the application of fluorescent steroid (Fig. 5). We found that in all cases, the onset of fluorescence was more rapid near the membrane ($\tau = 1.1 \pm 0.2$ s), followed by a slower increase intracellularly ($\tau = 3.3 \pm 0.5$ s, $P < 0.01$, paired *t* test relative to peri-membrane). The onset of fluorescence increase in the two compartments typically occurred simultaneously at our ability to resolve, indicating rapid movement from peri-membrane regions to intracellular compartments. The rise of intracellular fluorescence usually continued while the peri-membrane fluorescence decreased slightly from the maximum achieved during the period of fluorescent steroid application (Fig. 5). Because application of fluorescent steroid was brief (2 s), the decrease in peri-membrane fluorescence could

result from multiple factors, including diffusion into the extracellular solution devoid of steroid, redistribution into intracellular compartments and/or bleaching. However, the continued growth of intracellular fluorescence in the absence of continued extracellular resupply strongly suggests that redistribution explains at least part of the peri-membrane fluorescence loss. Although our methods do not allow us to definitively distinguish membrane fluorescence from cytoplasmic fluorescence, the ability to resolve two separate fluorescence pools makes it likely that we are examining fluorescence related to plasma membrane and intracellular reservoirs. In summary, these imaging results directly support the hypothesis that redistribution to intracellular compartments participates in the time course of onset and apparent potency of steroid effects at GABA-A receptors.

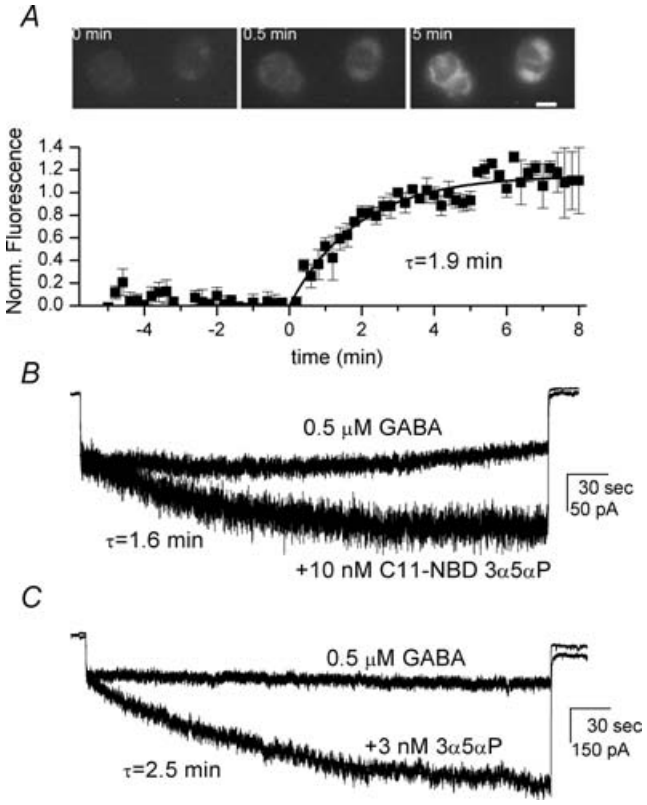


Figure 4. Accumulation of fluorescent steroid follows the time course of potentiation
A, fluorescence images from untransfected HEK cells at time zero (before application of fluorescent steroid analogue; left panel), 30 s after addition of 5 nM C11-NBD 3α5αP (centre) and 5 min after continuous exposure (right). Scale bar indicates 20 μm. Lower panel gives the summary of the change in fluorescence from 3 independent fields of cells imaged every 12 s. Points indicate mean $\pm$ standard error of the mean. The increase in fluorescence was fitted with an exponential function with a time constant of 1.9 min (continuous line). *B*, macroscopic responses from a HEK cell expressing $\alpha1\beta2\gamma2$L GABA-A receptors. The cell was exposed to 0.5 μM GABA or GABA + 10 nM C11-NBD 3α5αP. The application duration was 6 min. The slow phase in the GABA + steroid curve was fitted with an exponential function with a time constant of 1.6 min. Application of 10 nM C11-NBD 3α5αP alone did not result in appreciable current responses even after 5 min applications (not shown). *C*, macroscopic responses from a HEK cell expressing $\alpha1\beta2\gamma2$L GABA-A receptors. The cell was exposed to 0.5 μM GABA or GABA + 3 nM 3α5αP. The application duration was 6 min. The slow phase in the GABA + steroid curve was fitted with an exponential function with a time constant of 2.5 min. Application of 3 nM 3α5αP alone did not result in appreciable current responses even after 5 min applications (not shown).

Discussion

In this characterization of steroid potentiation kinetics, we have examined the underlying mechanism for slow potentiation by an endogenous neurosteroid 3α5αP, and the role of intracellular compartments in modulating the time course and dose-dependency of steroid potentiation. The data suggest that upon steroid application extracellularly, steroid equilibrates between the cell membrane and intracellular compartments, resulting in an initial depletion of steroid from the cell membrane. This strongly contributes to the time courses of current traces and estimated concentration dependence of potentiation because the steroid binding sites are formed by residues in the transmembrane regions of the receptor (Hosie *et al.* 2006), and the concentration of steroid in

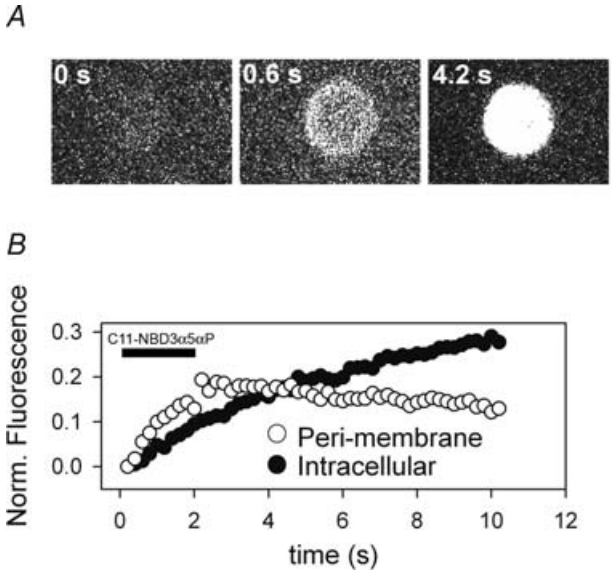


Figure 5. Direct evidence for compartmentalization of steroid
A, images from an experiment in which 0.5 μM C11-NBD 3α5αP was rapidly applied by perfusion to an untransfected HEK cell for 2 s (beginning at time 0), followed by return to static bath. Images were acquired every 200 ms, and representative time points are shown in the upper left of each image. Steroid accumulated most prominently initially around the periphery of the cell, followed by slower intracellular accumulation. The cell diameter was 22.1 μm. *B*, time course of fluorescence accumulation from the cell in panel *A*. A curved peri-membrane region of interest 8 μm long and 1.4 μm wide was used to quantify peri-membrane fluorescence. A circular region of interest of 2.9 μm diameter placed near the cell centre was used to quantify intracellular fluorescence. The bar denotes the application of C11-NBD 3α5αP to the cell.

the membrane, not in the aqueous phase, determines the degree of potentiation.

When the steroid 3α5αP was coapplied with GABA, the whole-cell current response contained two phases. The fast component had an amplitude and time constant similar to those seen in response to GABA alone. The second phase was more slowly developing, and was affected by the concentration of steroid, becoming more rapid as the steroid concentration was elevated. A preincubation with steroid abolished the slow phase, and the slow phase of current development was missing in excised outside-out patches. Furthermore, the time courses for channel potentiation and accumulation of fluorescence by a low concentration of labelled steroid C11-NBD 3α5αP were similar, cumulatively suggesting that steroid equilibration between the cell membrane and intracellular compartments underlies the slow current development.

The use of fluorescently labelled, pharmacologically active steroid analogues has allowed us to examine the time course of the spatial distribution of steroids under similar conditions to the physiological studies. When a low concentration of analogue is applied, the total cellular accumulation occurs with a similar time course to the development of potentiation. This suggests that the plasma membrane pool of analogue, which interacts with the GABA-A receptor, is at equilibrium with the total cellular pool under these conditions. However, when a briefer (2 s) pulse of a higher concentration of analogue is applied, the fluorescence at the margins of the cell (including the plasma membrane and submembranous structures) increases more rapidly than that in the cell interior. Because analogue is only added to the external leaflet, this suggests that the redistribution to other regions of the cell occurs at a slower rate than addition to the plasma membrane, under these conditions. These observations are only suggestive, as the structures and processes involved in the redistribution are at present unknown and are likely to be complex, and much further work will be required to quantitatively measure redistribution rates. However, the observations clearly indicate that at high concentrations of applied steroid it is possible to load the cell membrane relatively rapidly, while at lower concentrations the inferred membrane concentration tracks the overall cellular accumulation of steroid. This is clearly consistent with the hypothesis that redistribution of steroid can affect the time course and extent of neurosteroid action.

In cell-attached single-channel recordings, where 3α5αP was coapplied with GABA through the pipette solution, the addition of steroid to the bath had a remarkable effect on channel activity, allowing potentiation at steroid concentrations previously deemed ineffective. We propose that under normal experimental conditions (i.e. without steroid in the bath), efficient steroid diffusion from the patch membrane to the intracellular compartments, and possibly to the rest of the cell membrane outside the patch, reduces the concentration of steroid in the patch membrane. The addition of steroid to the bath leads to a uniform distribution of steroid throughout the cell compensating for the otherwise mostly unidirectional diffusion of steroid out of the patch membrane.

The work presented here was carried out on an endogenous neurosteroid 3α5αP. Qualitatively similar results were obtained with another endogenous neurosteroid (3α,5β)-3-hydroxypregnan-20-one, and with several synthetic neuroactive steroids (data not shown). This suggests that the findings are relevant to most steroids and, possibly, other lipophilic modulators.

These findings have strong relevance to elucidating the physiological role of endogenous steroids. Although several studies have found evidence suggesting that normal levels of endogenous neurosteroids can influence the functioning of native GABA-A receptors (Belelli & Herd, 2003; Puia *et al.* 2003; Keller *et al.* 2004), in many cases the free steroid concentrations in the CSF are believed to be at levels below the threshold at which they act on the GABA-A receptor in typically used experimental approaches. The EC_{50} for 3α5αP actions on the $\alpha1\beta2\gamma2$ receptor is 100 nM or more (Maitra & Reynolds, 1998; Shu *et al.* 2004; Weir *et al.* 2004; Akk *et al.* 2005). In contrast, the total concentration of 3α5αP ranges from 1–2 nM to 15–20 nM in different brain regions (Cheney *et al.* 1995; Weill-Engerer *et al.* 2002). Although other, more sensitive subunit combinations exist in the brain (Wohlfarth *et al.* 2002), and the steroid levels themselves are known to increase in response to stress and other factors (Purdy *et al.* 1991; Finn & Gee, 1993; VanDoren *et al.* 2000; Smith, 2001), the existing concentration–effect data would seem to exclude the involvement of the $\alpha1\beta2\gamma2$ GABA-A receptor in steroid actions under normal conditions. Our findings, however, indicate that under typical physiological conditions where the cells are pre-equilibrated with the steroid, instead of being periodically exposed to high steroid doses, at least some modulation of the $\alpha1\beta2\gamma2$ receptor should be expected.

We are unable to predict to what degree native GABA-A receptors are modulated by endogenous neurosteroids under physiological conditions. Certainly, the fact that Althesin (a mixture of the steroids alphaxalone and alphadalone) and other exogenously applied steroidal agents are able to elicit anaesthesia, presumably via interaction with the GABA-A receptor, suggests that modulation by endogenous steroids is not saturated. Synthesis and breakdown of steroids may also influence the local steroid levels (Belelli & Herd, 2003; Agis-Balboa *et al.* 2006). The preference for steroids to be retained by intracellular reservoirs makes it likely that a prominent effect of endogenous steroid action may be autocrine (Agis-Balboa *et al.* 2006), although a strong source of overall CNS neurosteroid levels may be from the periphery (Purdy *et al.* 1991). Finally, the concentration

of the most relevant steroid, the cell membrane-localized steroid, which is likely to be in rapid equilibrium with the binding sites on the GABA-A receptor, remains unknown. These considerations combine to make it difficult to predict the degree of neurosteroid tone at GABA-A receptors from overall measurements of steroid concentrations.

Our results have another, more practical implication for studies using steroids or other lipophilic compounds. Macroscopic electrophysiological measurements are typically conducted by drug applications of finite duration. The results presented in this work show that the duration of drug application has a profound effect on the degree of modulation observed because of the time required to allow full equilibration of the drug between the cell membrane and intracellular compartments.

Similarly, single-channel measurements in the cell-attached configuration may be influenced by steroid diffusion to other lipid compartments such as other portions of the cell membrane or intracellular membrane compartments. Although in the case of cell-attached recordings, the cell, or more precisely, the patch membrane, is exposed to the drugs for long periods of time (typically, several minutes), this may not be sufficient to fill the vast intracellular volumes accessible to the neurosteroid. In any case, diffusion of the steroid from the pipette seems to be insufficient to compensate for the outmigration of steroid from the patch membrane.

Is the redistribution of neurosteroids between the cell membrane and intracellular compartments accomplished through active or passive transport? Certainly, cholesterol has been shown to move between the cell membrane and various intracellular compartments in a process utilizing carrier proteins (Maxfield & Menon, 2006) and the entry of steroid hormones such as 17β-oestradiol or 25-(OH) vitamin D3 into the cell involves a cell surface localized facilitator protein, megalin (Christensen & Birn, 2002). It is not clear, however, whether either of these processes, facilitation of entry into the cell and intracellular trafficking, applies to neurosteroids, and too many unknown parameters preclude even a qualitative assessment of whether active transport could be sufficiently fast to deplete the cell membrane of steroid during a brief steroid application. Intracellular membrane compartments may be located quite close to the plasma membrane or even be continuous with it (Stan, 2005; Levine & Loewen, 2006), and in any case a recent modelling study showed that free diffusion of steroid hormones progesterone, testosterone and oestradiol through the membrane can be extremely rapid (Oren *et al.* 2004).

Our observations are consistent with a series of recent studies that have demonstrated that steroids have relatively slowly developing and reversing actions. Studies of dissociated hippocampal neurons in primary cultures have shown remarkably slow onsets for either potentiation or direct gating by low concentrations of steroids (Shu *et al.* 2004). The use of fluorescently tagged neurosteroid analogues has demonstrated that these drugs reach intracellular pools, and that the intracellular pools can supply drug to the plasma membrane (Akk *et al.* 2005). The present findings extend these studies by providing evidence that the slow actions are not likely to result from slow kinetics of interaction with the GABA-A receptor, but rather reflect the slow equilibrium of steroid in a membrane compartment that is in equilibrium with the receptor. This explanation also raises the possibility that the time course of action, and the apparent affinities, of steroids may depend on the nature of the cell expressing the receptor, as the relative sizes of the cellular compartments may vary, as well as the equilibration rates among them. In future studies it will be interesting to examine whether modulation of synaptic transmission in brain slices is similarly dependent on the experimental protocol.

Recently, Wegner *et al.* (2007) showed that repetitive steroid (alphaxalone or THDOC) applications lead to a progressively greater potentiation, a phenomenon which at first glance appears to share some similarities with the one described in the present study. The effect was observed with many recombinant receptor types as well as native receptors in human NT2 teratocarcinoma cells, and was found to be dependent on phosphorylation. Our results showing a strong correlation between the rate of potentiation of GABA currents and the rate of accumulation of intracellular fluorescence in response to low concentrations of steroids suggest that second messengers are unlikely to determine the effects we have observed. However, it is possible that phosphorylation of cellular targets may affect steroid movement into the cell and, consequently, channel potentiation.

To recapitulate, we present findings suggesting that steroid movement between the cell membrane and intracellular domains can affect GABA-A receptor modulation, presumably via a decrease in the concentration of membrane-localized steroid. The findings indicate that when steroid movement out of the patch membrane is compensated by bath-applied steroid, channel potentiation is observed at much lower concentrations, implying that physiological concentrations of neurosteroids may be sufficient to produce modulation of GABA-A receptors *in vivo*.

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